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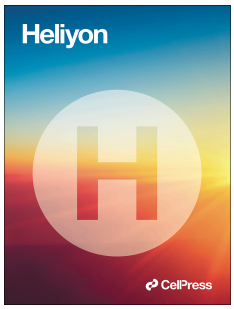

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Evaluation of neutralizing antibody titers against SARS-CoV-2 JN.1 Omicron subvariant during pregnancy - A case series study

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Abstract: Background: SARS-CoV-2 infection during pregnancy poses health risks to both mother and fetus. This study investigates neutralizing antibodies (NAbs) against the SARS-CoV-2 JN.1 Omicron subvariant in pregnant women, focusing on responses to natural infection, vaccination, and passive immunity. **Methods:** A single-center, prospective study collected blood samples from 19 pregnant women at various pregnancy stages and postpartum. NAb titers were analyzed using a pseudovirus neutralization assay, with statistical analyses (p-value <0.05) conducted using unpaired t-test with Welch's correction. **Results:** Among participants, 63.2% had at least one positive NAb titer, with only one vaccinated case. No significant difference in NAb titers was found between symptomatic and asymptomatic women. NAbs were detected in cord blood, especially when infection or vaccination occurred close to delivery, indicating passive immunity transfer to the newborn. **Conclusion:** NAb titers change dynamically during pregnancy, increasing then decreasing. Most pregnant women were asymptomatic and NAbs were effectively transferred to the fetus when infection or vaccination occurred near delivery. These findings highlight the importance of vaccination timing, suggesting late second or third trimester vaccination may provide better protection, emphasizing the need for adherence to vaccination guidelines to optimize maternal and neonatal immunity.

Keywords: Maternal immunity; neutralizing antibodies; passive immunity; SARS-CoV-2; pregnancy

1. Introduction

SARS-CoV-2 infection during pregnancy raises significant concerns regarding the health risks to both the mother and the fetus, making the study of COVID-19 in this population particularly important.[1] Understanding the immune response of pregnant women, and especially the level of production and efficacy of neutralizing antibodies (NAbs), either acquired by natural infection or vaccination against COVID-19, is a significant area of research.[2] NAbs play a crucial role in the defense against viral infections by inhibiting the virus ability to enter host cells and replicate.[3, 4] The generation of these antibodies following infection or vaccination is an essential aspect of the immune response, providing insight into both individual and population-level immunity.[4] During pregnancy, the maternal immune system undergoes adaptations to tolerate the fetus, which might alter the response to infections.[5, 6] Several factors influence the immune response to SARS-CoV-2 during pregnancy, including the trimester of infection, the severity of the disease, and the individual's health condition.[7, 8] Additionally, the transplacental transfer of antibodies from the mother to the fetus is of paramount importance, as it can confer passive immunity to the newborn, offering protection during the early months of life when the immune system is still developing.[9, 10] For instance, infections occurring during the third trimester might result in a higher likelihood of having a sufficient antibody level at the time of delivery, promoting adequate transfer to the fetus, when compared to earlier in pregnancy.[11] The severity of the disease also plays a role, with more severe cases potentially leading to higher antibody titers.[4] Moreover, some studies have suggested that pregnant women might experience more severe COVID-19 outcomes compared to non-pregnant women, though the data are not entirely consistent.[8, 12]

Vaccination against SARS-CoV-2 during pregnancy has been shown to be safe and effective. Even though the Center for Disease Control (CDC) points out that there are no robust data to support one trimester over the other, pregnant women who receive COVID-19 vaccines in the late

second or the third trimester generally generate robust neutralizing antibody responses. This can enhance protection for both the mother and the fetus.[9, 13]

Understanding the levels and persistence of these antibodies can inform strategies for managing pregnant women during the pandemic, including decisions about vaccination timing and the need for booster doses. Moreover, this research has implications for the health of the fetus and newborn, as higher antibody titers in the mother could potentially lead to optimal protection for the infant. This study aims to evaluate the role of neutralizing antibodies (Nabs) in protecting pregnant women against SARS-CoV-2, particularly assessing maternal-to-fetal transfer of antibodies

2. Materials and Methods

2.1. Study design

This single-center, prospective, and longitudinal study, focuses on biological investigations. One of the secondary objectives is to better understand the passive immunity against SARS-CoV-2 in newborns from vaccinated or infected mothers. The study involves blood sample collection at various pregnancy and postpartum stages, in accordance with standard care: at inclusion ([4-8] weeks), and subsequently at [3-4]-, [6-7]- and [8-9]- months of pregnancy, delivery day+1, and 3 weeks post-partum. Healthy pregnant females aged 18-50 years were recruited, regardless of their SARS-CoV2 positive status Main exclusion criteria were hepatic or renal insufficiency, smoking more than 15 cigarettes/day, drinking alcohol and suspected or confirmed immunodeficiency.

2.2. Sample collection

The study was approved by the local ethic committee of CHR Huy (N° CE035 10/2022). The study complies with the Declaration of Helsinki, ensuring informed consent was obtained from all participants, with ethical guidelines strictly followed to protect patients' privacy. Written informed consent as well as information form were obtained from all participants. Blood samples were collected at specific time points defined by the standard of care of the hospital. Samples were collected from 25th May of 2023 to 7th May of 2024. Blood was taken by venipuncture in the antecubital vein and collected into Clot Activator Tube (CAT) containing silica (BD® vacutainer, Novolab, Belgium), using a 21-gauge needle. Serum was obtained from the supernatant fraction of blood tubes after at least 30 min upright rest at room temperature, followed by a centrifugation for 10 min at 1500 *x g*. All sample were treated and frozen at -20°C within 4 hours. Additionally, umbilical cord blood was collected and processed immediately after birth for participants included in the sub study. The collected samples were thawed at 37°C in a water bath prior to analyses.

2.3. Analytical procedure

HEK-293T hACE2 cells (RRID: CVCL_0063) were seeded at a density of 8500 cells per well in a white 384-well cell culture plate from Greiner Bio-One (Kremsmünster, Austria). Authentication of HEK-293T hACE2 cells was performed following standard protocols, and they were tested to confirm absence of mycoplasma contamination. The sera were heat-inactivated by incubation in a water bath at 54°C for 30 minutes. Following inactivation, the sera were serially diluted in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), purchased from Lonza (Bâle, Switzerland). The diluted serum samples were mixed in a 1:4 ratio with Omicron JN.1 pseudovirus particles obtained from E-enzyme (Gaithersburg, MD, USA), which are replication-deficient MLV particles pseudo typed with the SARS-CoV-2 spike protein. This mixture was incubated for 2 hours at 37°C to allow for the interaction between the antibodies in the serum and the pseudovirus. Following the incubation period, 17.5 µL of the mixture and 7.5 of DMEM were added to the HEK-293T hACE2 cells and the plates were incubated for an additional 48 hours at 37°C. After this incubation, 20 µL of luciferase assay reagent from the FireFly Luciferase Kit (E-enzyme, Gaithersburg, MD, USA) was added to each well to measure the luciferase activity. This activity is proportional to the number of cells infected by the pseudovirus. The luminescence signal was read using a Spectramax iD3 analyzer (San Jose, CA, USA), and the raw data, expressed in relative luminescence units (RLU), were normalized to a positive control where cells were incubated with the pseudovirus in the absence of serum. The antibody titer was determined as the serum dilution at which 50% of the infectivity was inhibited (IC50), using a non-linear sigmoid regression model. Samples with a titer below 1:20 were considered negative. This approach ensures precise quantification of the NAb response in the analyzed serum samples. This pseudovirus technique has demonstrated good reproducibility and robustness in the numerous publications in which it has been involved. [14-16]

2.4. Statistical analyses:

The normality of the distribution was assessed using the Anderson-Darling test after log-transformation of the data. Descriptive statistics were computed by using mean ± standard deviation (± SD). Differences regarding age and NAbs titers, between positive and negative group were analyzed using an unpaired t test where the Welch's correction was applied when SDs were not equal. A p-value of less than 0.05 was considered significant. Statistical analyses were performed using GraphPad Prism version 10.2.0 (GraphPad Software, MA, USA).

3. Results

3.1. Study population

A total of 19 patients were recruited and all were analyzed. The positivity was characterized by at least one positive neutralizing antibody titer, irrespective of the time point. In this longitudinal study, the number of samples was not consistent at each time point (i.e., [4-8] weeks: n=10, [3-4] months: n=18, [6-7] months: n=16, [8-9] months: n=14, delivery day +1: n=7 and 3 months postpartum: n=1). Among all the population of these pregnant women, 12 (63.2%) showed at least one positive neutralizing antibody titer (i.e., ≥20). The mean age in the positive and the negative group were similar (i.e., 31±4.5 vs 31±4.7 years old, respectively, p value>0.99). Among women revealing positive testing, only 1 has declared to have received vaccination with BNT162b2 adapted booster, at 3 months pregnancy. As 12 women had at least one positive result, and only one had received a vaccination, the remaining 11 had therefore been infected. As only one participant in this study was vaccinated, no conclusions can be drawn regarding vaccine-induced immune responses, and this data is included solely for completeness.

3.2. Neutralizing antibody titers

The **Figure 1** shows the neutralizing antibody titers, for each infected woman at each available time point, expressed in weeks of pregnancy. Surprisingly, among the 11 women that were infected, only 2 (18.2%) declared symptoms while the 9 others did not express any symptom of infection. No significant difference was observed regarding their antibody titers (unpaired t-test Welch's correction p value = 0.2805). Interestingly, the mean age of symptomatic women was higher when compared to the asymptomatic women (33±4.2 vs 30±3.6 years old), but this difference was not significant (unpaired t-test p value=0.27). However, as the symptomatic group only contains two women, reliable comparison is difficult.

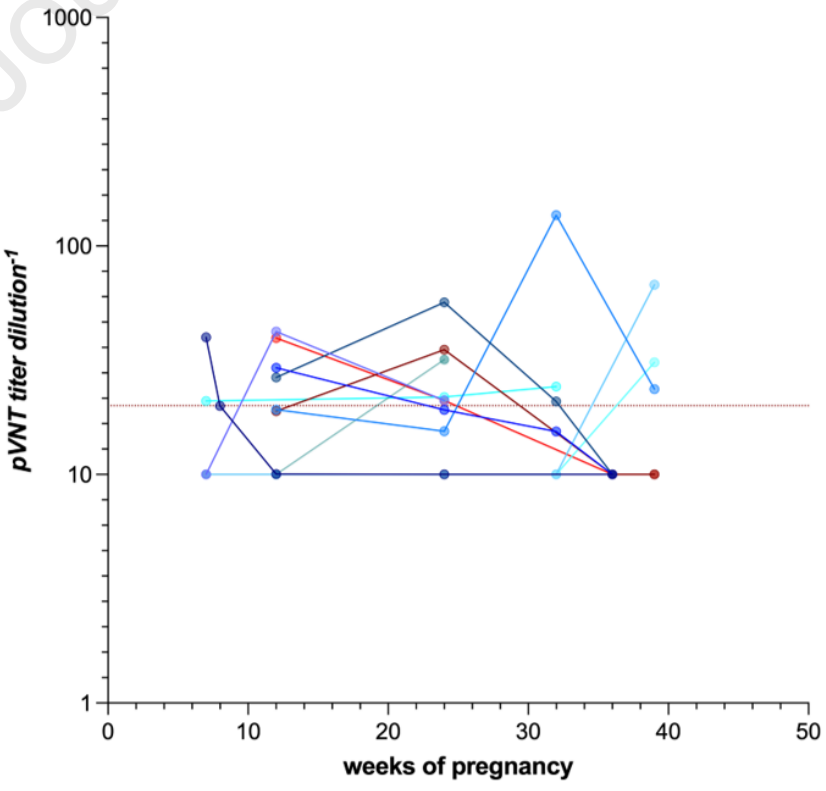

Figure 1. Neutralizing antibody titers, for each infected woman at each available time point, expressed in weeks of pregnancy. The nuances of blue represent the asymptomatic while the nuances of red show the symptomatic patients. The orange dotted line symbolizes the threshold for positivity.

3.3. Antibody transmission

Cord blood was only collected for 7 of the patients with positive titer (**Table 1**). The neutralizing antibody titer of the mother at the time of delivery was compared to the titer obtained from the cord blood. Interestingly, only one patient with a positive NAbs titer at 6 months pregnancy, was linked to a cord blood's positive NAbs titer (i.e., >20). The closer the infection or vaccination is to delivery, the higher the antibody titer is. This estimation plot in **Figure 2**, shows the mother's NAbs titer at delivery and its related cord blood's Nabs titer. There is close to no difference between the mother's and the cord blood's titer (unpaired t-test p value = 0.34). These results indicate a rapid and "real-time" transfer of antibodies. The mother's antibody levels are virtually identical, at the corresponding collection time, to the levels found in the cord blood (**supplementary material figure 1**). Furthermore, these results show little persistency of neutralizing antibodies in the cord from infected pregnant women.

Table 1. Neutralizing antibody titers of the mother and of the cord blood, at delivery. Positive (P) or Negative (N) titer correspond to a Neutralizing antibody titer of the cord blood above (Positive) or below (Negative) the threshold. The trimester of the positive point correspond to the time point were the mother express the higher antibody titer.

Neutralizing antibody titers at delivery day + 2	***Neutralizing antibody titers of*** **the cord blood**	**Positive (P) or Negative (N) titer**	**Trimester of the positive point**
10	10	N	1st
10	20.08	P	2nd
23.63	21.08	P	3rd
67.73	32.19	P	3rd
10	10	N	2nd
30.92	22.64	P	3rd
10*	10	N	1st
*This patient was vaccinated			

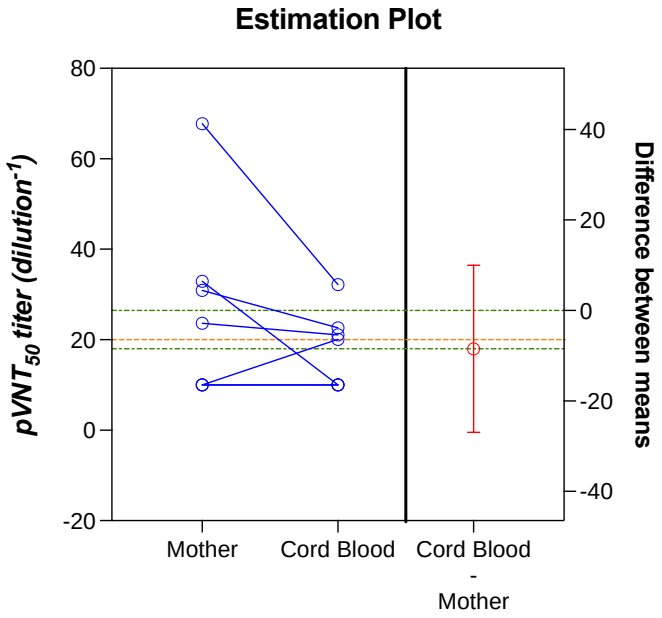

Figure 2. Estimation plot of the unpaired t-test. The mother's pVNT titer closer to delivery is related to it corresponding cord blood s 'titer. The orange dotted line represents the positive threshold. The right panel shows the difference between the means ± its 95% confidence interval (95% IC). The green dotted line symbolizes the upper and lower confidence limit show how large and small the effect size could be. These are established as Y=0 on the right axis is aligned at the position of the mean of the first group plotted on the left axis (i.e., mother). The lower limit is aligned at the position of the mean of the second plotted group (i.e., cord blood).

4. Discussion

This study aligns with existing literature that highlights the dynamic changes in neutralizing antibody (NAb) titers during pregnancy. We observed, through longitudinal sampling of infected pregnant women, a progressive increase in NAb titers followed by a rapid decrease. While this study focused on antibody-mediated immunity, emerging evidence supports the critical role of T-cell immunity in COVID-19 protection, particularly in reducing severity. T cells play a pivotal role in the adaptive immune response to SARS-CoV-2 by mediating targeted cellular immunity. CD8+ cytotoxic T lymphocytes (CTLs) recognize and lyse infected host cells that present viral peptides via MHC class I molecules, thereby controlling viral replication within infected tissues. Concurrently, CD4+ helper T cells enhance the immune response by promoting B cell maturation and differentiation into plasma cells, leading to robust antibody production, and by assisting CD8+ T cell expansion and function. Moreover, SARS-CoV-2-specific memory T cells persist post-infection, providing a rapid recall response upon subsequent exposures to the virus, which is crucial for long-term immune protection and may mitigate disease severity in reinfections. [17, 18] Future studies examining both humoral and cellular immunity would provide a more comprehensive understanding of immune protection in pregnant population. In this study, two of the eleven pregnant patients with SARS-CoV-2 infection were symptomatic, which is less than the study of the National Institutes of Health (NIH) which found that roughly 47% of pregnant women with COVID-19 were asymptomatic. Moreover, Khan et al. (2021) reported in their meta-analysis that a substantial number of SARS-CoV-2 infected pregnant women were asymptomatic, highlighting the variability in symptom presentation among this population. [19]

The detection of NAbs in cord blood, particularly when the infection occurred close to delivery, is consistent with other studies that have demonstrated effective transplacental transfer of antibodies.[20, 21] Moreover, the same results were observed with one particular patient of the study, but during a previous pregnancy.[22] This transmission provides the newborn with passive immunity, which is crucial during the early months of life when the infant's immune system is still developing. Investigations by Gray et al. (2021) and Collier et al. (2021) has shown that maternal SARS-CoV-2 infection can result in the presence of NAbs in cord blood, highlighting the potential for neonatal protection through passive immunity.[23, 24] Additionally, the efficiency of antibody transfer is higher in the third trimester, as reported by Rottenstreich et al. (2022), who found that timing of infection significantly affects the levels of antibodies transferred to the fetus.[25] This is crucial for informing vaccination strategies and ensuring optimal protection for both mother and child during the critical perinatal period. Moreover, in our study, even though we observed a transplacental transfer, we demonstrated its low efficiency as the cord blood's titer struggled to reach the positivity threshold. This may be due to the variant analyzed in this study, JN.1, which showed significant immune escape compared with the Wild Type strain. Favresse et al. reported in their study an average titer 14.43 times lower for the JN.1 variant.[26] The JN.1 variant was chosen for its prevalence during the study period; however, testing across multiple SARS-CoV-2 strains, particularly earlier variants, could yield further insights on the efficacy of transferred antibodies. Gillot et al. highlighted the reduction of neutralizing capacity as function of the evolution of SARS-CoV-2, in their study they reported 27.0% of neutralizing capacity against KP.3 (one of the latest variants under monitoring according to World Health Organization) compared to 47.0% for JN.1. Such differences could be observed in our pregnancy cohort. [15]

In addition to the trends observed in naturally infected pregnant women, the impact of SARS-CoV-2 vaccination during pregnancy presents a different perspective on NAb titers. Vaccinated pregnant women typically exhibit higher and more consistent levels of NAbs compared to those who acquire immunity through natural infection. This is particularly significant as higher NAb titers are associated with better protection against COVID-19 and its severe outcomes. [27, 28] Studies have shown that vaccination during pregnancy not only induces robust maternal immune responses but also enhances the transplacental transfer of antibodies to the fetus.[29, 30] For instance, Munoz et al. (2023) demonstrated that COVID-19 booster vaccination during the third trimester of pregnancy resulted in significantly higher NAb levels in both maternal and cord blood compared to those observed in unvaccinated, naturally infected pregnant women.[9, 31] This suggests that the timing and nature of immune exposure (vaccination versus infection) play crucial roles in determining antibody levels and subsequent immunity. Moreover, our results do not support the idea of CDC that the timing of vaccination is not of great importance. Indeed, we demonstrated that an increased in Nabs transmission is only observed in the cord blood when the mother is infected in her late second/third trimester. In the future, definition of a more precise time point for vaccination could reduce significant complication of COVID-19 infection in newborns.

Moreover, according to Favresse et al., the rate of negative individuals 6 months after vaccination is over 50% it is therefore logical that our patient will no longer have detectable NAbs in the long term.[26] This also highlight the ineffectiveness of current vaccines, which are unsuited to the strains in circulation and therefore unable to provide a sustained immune response over time. However, this study not able to fully demonstrate these assumptions and draw definitive conclusion as it has a limited sample size. Furthermore, only one woman underwent vaccination.

One limitation of our study is its focus on neutralizing antibodies alone. Binding antibodies, measurement could offer additional insights, especially regarding immune strength and durability in pregnant populations. However, Douxfils et al. demonstrated a poor correlation between the measurement of binding antibodies and NAbs, with a specificity of only 33.33% (95% CI:15.63 - 55.32%). [32] Moreover, studies highlighted the importance of Nabs instead of binding antibodies as correlate of protection against infection [16, 33].

When evaluating immune responses, it is essential to consider individual health conditions and other factors that might influence. For instance, hypertension and diabetes can modulate the effectiveness of both natural infection and vaccination by causing chronic inflammation and altering immune system responses, which can impair the body's ability to produce a robust and effective immune response.[34, 35] These conditions might affect the antibody response and its transfer to the fetus, necessitating personalized approaches to vaccination and maternal care.

5. Conclusions

Our results demonstrate a progressive increase followed by a decrease in NAb titers in pregnant women, with most participants remaining asymptomatic and unaware of their infection. Additionally, our findings indicate a dynamic change in Nab titers during pregnancy, highlighting the maternal-to-fetal transfer of antibodies close to delivery. These findings underscore the important of understanding natural immune responses during pregnancy and their role in neonatal protection. This study cannot draw conclusion regarding the potential of optimizing vaccination strategies due to the limited number of vaccinated patients. However, it emphasizes the importance of tailored vaccination timing, especially in the later stages of pregnancy, to maximize neonatal immunity. Further research with vaccinated cohorts integrating both antibody and T-cell data is essential to better inform vaccination guidelines.

Supplementary Materials: Figure S1: Spaghetti plot representation of the mother's Nabs titer (blue). The Nabs titer of the cord blood is represented in green.

Author Contributions: Conceptualization, M.D.; methodology, M.D..; software, M.D.; formal analysis, M.D. and C.G..; investigation, C.G. and E.C..; resources, J.D..; data curation, M.D..; writing—original draft preparation, M.D. and C.G..; writing—review and editing, C.D., E.C., J.D., J.F. and L.M..; supervision, J.D.; project administration, M.D.; funding acquisition, J.D.. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of CHR Huy (N° CE035 10/2022).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Conflicts of Interest: The authors declare no conflicts of interest.

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Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: